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Title: Tomatoes Having Reduced Polygalacturonase Activity Caused by Non-Transgenic Mutations in the Polygalacturonase Gene.

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FIELD OF THE INVENTION

This invention concerns mutations in the fruit polygalacturonase (PG) gene of tomato. This invention further concerns tomato plants having mutations in their PG genes. This invention further concerns a method that utilizes non-transgenic means to create tomato plants having mutations in their PG genes.

BACKGROUND

United States consumers spend more than $4 billion each year on fresh market tomatoes. During the summer months, most of these fresh market tomatoes are grown on farms located throughout the United States and then sold locally. During the cooler months, when locally grown tomatoes are not available, most of these tomatoes are grown in the southern portions of the United States and in Mexico and then shipped by truck throughout the rest of the country. Unfortunately, when these southern grown tomatoes are allowed to fully ripen on the vine before shipping, they do not remain in marketable condition long enough for supermarkets to shelve them and for consumers to buy them.

To prevent the tomatoes from rotting before they reach consumers, farmers typically pick, pack, and ship the tomatoes while green. Before sale, the green tomatoes are gassed with ethylene to redden them. These unripened "gassed" tomatoes do not spoil quickly, but they have developed a reputation for poor flavor, especially compared to the summer "vine-ripened" tomatoes.

Due to consumer dissatisfaction with the unripened "gassed" tomatoes, research and breeding efforts have focused on developing tomatoes that exhibit a longer shelf-life when they are allowed to ripen fully on the vine. One approach to developing longer shelf-life tomatoes is to use traditional breeding techniques, i.e., crossing tomato plants with desired characteristics and selecting those progeny plants with fruits exhibiting longer shelf-lives. While traditional breeding techniques have been used to develop most of the tomato cultivars used by growers today, these methods are very time intensive. It can take years to breed a novel tomato variety that may exhibit only a modest increase in shelf-life.

Another approach to developing longer shelf-life tomatoes is to use genetic techniques to manipulate the biochemical and physiological changes associated with the ripening process in tomatoes. One biochemical change in ripening fruit is the depolymerization and solubilization of cell wall polyuronides by the ripening-induced cell wall degrading enzyme, polygalacturonase (PG). Tomato fruit PG (Della Penna et al., Proc. Natl. Acad. Sci. U.S.A. 1986 83:6420-6424; Bird et al., Plant Mol. Biol. 1988 11:651-662) belongs to a family of tomato PG genes. PG enzyme activity increases dramatically during the ripening of many fruits, including tomato, and is the primary enzymatic activity responsible for cell wall polyuronide degradation.

For example, in U.S. Patent Nos. 5,107,065; 5,442,052; 5,453,566; 5,569,831; and 5,759,829, tomato plants were transformed with DNA constructs encoding an antisense oligonucleotide for the PG gene. When expressed, the foreign DNA provided an RNA sequence capable of binding to the naturally existing mRNAs of the PG gene in the transformed tomato plant thereby preventing the translation of the mRNA into the PG protein. The fruit of transformed tomato plants showed improved properties in terms of slower softening post harvest, thereby increasing the shelf-life of the tomato.

Another research group, using a complicated series of transgenic manipulations involving transposon sequences from another plant species, created a "knock out" of the PG gene in tomato. Enzymatic analysis of fruit from plants containing the knock out of the PG gene showed at least a 1000-fold reduction in PG levels. See Cooley, M. B. and Yoder, J.I., *Plant Mol. Biol.*, 1998 Nov. 1, 38(4):521-30; Cooley et al., *Mol. Gen. Genet.* 1996 Aug. 27, 252(1-2):184-194.

This anti-sense and "knock-out" work indicates that fruit PG gene expression is not necessary for viable, normal tomato fruit production. While several features of the ripening process remain normal, transgenic tomatoes having reduced PG gene expression exhibit slower softening post harvest and increased shelf life. Additionally, these transgenic tomatoes exhibit a lower incidence of post-harvest disease infection due to the preservation of intact fruit skin and coat caused by the delayed softening. Therefore, the tomatoes with reduced PG have fewer cosmetic blemishes which deter customers.

Reduced PG enzyme activity is important not only to the fresh market tomato industry but also to the processed tomato industry. During commercial processing of tomatoes, pectin integrity of the tomato is lost by enzymatic degradation of the pectin by PG. In order to avoid this degradation, a rapid, high heat treatment is used to destroy the PG enzyme activity. The annual cost associated with the total energy required to bring millions of tons of tomatoes to a temperature sufficient to rapidly inactivate the PG enzyme is a significant cost to the tomato processing industries.

While the use of these genetic techniques has resulted in producing tomatoes with reduced PG gene expression, the genetic techniques used to date employ recombinant DNA being introduced into tomatoes. Since many consumers have clear preferences against genetically modified foods, it would be useful to have a tomato exhibiting reduced levels of fruit PG that was not the result of genetic engineering methods. However, to date, no one has ever found or described a naturally occurring "knockout" of the endogenous tomato PG gene. Therefore, a tomato with its fruit PG gene either knocked out or otherwise hindered would have tremendous value to the entire tomato industry.

SUMMARY OF THE INVENTION

In one aspect, this invention includes a tomato plant, tomato fruits, seeds, plant parts, and progeny thereof having reduced fruit polygalacturonase enzyme activity compared to the wild type tomato plants wherein the reduced fruit polygalacturonase enzyme activity is caused by non-transgenic mutation in the tomato fruit polygalacturonase gene.

In another aspect, this invention includes a tomato plant having tomato fruits which soften slower post harvest compared to wild type tomato fruits due to an altered polygalacturonase enzyme, as well as fruit, seeds, pollen, plant parts and progeny of that plant.

In another aspect, this invention includes food and food products incorporating tomato fruit having reduced polygalacturonase enzyme activity caused by a non-transgenic mutation in the fruit polygalacturonase gene.

In another aspect, this invention includes a tomato plant having reduced fruit polygalacturonase enzyme activity compared to the wild type tomato plants created by the steps of obtaining plant material from a parent tomato plant, inducing at least one mutation in at least one copy of a fruit polygalacturonase gene of the plant material by treating the plant material with a mutagen to create mutagenized plant material, culturing the mutagenized plant material to produce progeny tomato plants, analyzing progeny tomato plants to detect at least one mutation in at least one copy of a fruit polygalacturonase gene, selecting progeny tomato plants that have reduced fruit polygalacturonase enzyme activity compared to the parent tomato plant; and repeating the cycle of culturing the progeny tomato plants to produce additional progeny plants having reduced fruit polygalacturonase enzyme activity.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ. ID. NO: 1 shows the DNA sequence between the start and stop codons for the coding region of Polygalacturonase (Gen Bank Accession No. M37304).

SEQ. ID. No.: 2 shows the protein sequence encoded by SEQ. ID. No. 1.

SEQ. ID. NOS.: 3-46 show the DNA sequences for Polygalacturonase specific primers of the present invention.

SEQ. ID. No.: 47 shows the DNA sequence of the Polygalacturonase gene for Mutation 13345.

SEQ. ID. No.: 48 shows the protein sequence encoded by SEQ. ID. No. 47.

SEQ. ID. No.: 49 shows the DNA sequence of the Polygalacturonase gene for Mutation 13342.

SEQ. ID. No.: 50 shows the protein sequence encoded by SEQ. ID. No. 49.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is an illustration of the regions of the PG gene.

FIG. 2 is a LOGO analysis of Mutation 13345.

FIG. 3 is a LOGO analysis of Mutation 13342.

FIG. 4 is a graph of the results of a blind "squeeze" test.

FIG. 5 is a graph of the results of the DNS based assay for PG activity.

FIG. 6 is a composite graph of the results of the BCA based assay for PG activity.

FIG. 7 shows a Western blot of PG protein levels in Mutant 13345.

FIG. 8 shows a Western blot of PG protein levels in developing Wild Type Tomatoes.

FIG. 9 shows Western blots of PG protein levels in Mutants 13345 and 13342.

DETAILED DESCRIPTION

The present invention describes: a series of independent non-transgenic mutations created in the polygalacturonase (PG) gene of tomato; tomato plants having these mutations in their PG gene; and a method of creating and identifying similar and/or additional mutations in the PG gene of tomato plants. The present invention further describes tomato plants exhibiting reduced PG enzyme activity and slower fruit softening post harvest without the inclusion of foreign nucleic acids in the tomato plants' genomes.

As shown in **Fig. 1**, the tomato fruit PG gene (GenBank accession no. M37304) consists of nine exons **1** separated by eight introns **2**, and 5' and 3' untranslated regions. The DNA surrounding the gene regulates expression of the PG gene. The PG protein sequence contains eight highly conserved regions called blocks **3** (http://blocks.fhcrc.org/blocks-bin/getblock.sh?IPB000743), listed under IPB000773 at the Fred Hutchinson Cancer Research Center Blocks website. These regions are conserved amongst polygalacturonases from many organisms. Of all the conserved amino acid residues in the blocks, 15 amino acids are either invariant or are found in the majority of all polygalacturonases (using the criteria of only one other amino acid found at that position in a minority of protein sequences). J.G. Henikoff, *et al.*, *Nucl. Acids Res.* 28:228-230 (2000). S. Henikoff, *et al.*, *Bioinformatics* 15(6):471-479 (1999).

In order to create and identify the PG gene mutations and slower softening tomatoes of the present invention, a method known as TILLING was utilized. See McCallum, *et al., Nature Biotechnology* (April 2000), 18: 455-457; McCallum, *et al.,* (June 2000) *Plant Physiology*, Vol. 123, pp. 439-442; and US Patent No. 5,994,075,

all of which are incorporated herein by reference. In the basic TILLING methodology, plant material, such as seeds, are subjected to chemical mutagenesis, which creates a series of mutations within the genomes of the seeds' cells. The mutagenized seeds are grown into adult M1 plants and self-pollinated. DNA samples from the resulting M2 plants are pooled and are then screened for mutations in a gene of interest. Once a mutation is identified in a gene of interest, the seeds of the M2 plant carrying that mutation are grown into adult M3 plants and screened for the phenotypic characteristics associated with the gene of interest.

Any cultivar of tomato having at least one PG gene with substantial homology to Seq. I.D. No. 1 may be used in the present invention. The homology between the PG gene and Seq. I.D. No. 1 may be as low as 60% provided that the homology in the conserved regions of the gene are higher. Thus one of skill in the art may prefer a tomato cultivar having commercial popularity or one having specific desired characteristics in which to create their PG- mutated tomato plants. Alternatively, one of skill in the art may prefer a tomato cultivar having few polymorphisms, such as an in-bred cultivar, in order to facilitate screening for mutations within the PG gene.

In one embodiment of the present invention, seeds from the tomato plant are mutagenized and then grown into M1 plants. The M1 plants are then allowed to self-pollinate and seeds from the M1 plant are grown into M2 plants, which are then screened for mutations in their PG genes. However, one of skill in the art would understand that a variety of tomato plant materials, including but not limited to, seeds, pollen, plant tissue or plant cells, may be mutagenized in order to create the PG-mutated tomato plants of the present invention. However, the type of plant material mutagenized may affect when the plant DNA is screened for mutations. For example,

when pollen is subjected to mutagenesis prior to pollination of a non-mutagenized plant, the seeds resulting from that pollination are grown into M1 plants. Every cell of the M1 plants will contain mutations created in the pollen, thus these M1 plants may then be screened for PG gene mutations instead of waiting until the M2 generation.

Mutagens creating primarily point mutations and short deletions, insertions, transversions, and or transitions (about 1 to about 5 nucleotides), such as chemical mutagens or radiation, may be used to create the mutations of the present invention. For example, but not limited to, mutagens such as ethyl methanesulfonate (EMS), methylmethane sulfonate (MMS), N-ethyl-N-nitrosurea (ENU), triethylmelamine (TEM), N-methyl-N-nitrosourea (MNU), procarbazine, chlorambucil, cyclophosphamide, diethyl sulfate, acrylamide monomer, melphalan, nitrogen mustard, vincristine, dimethylnitosamine, N-methyl-N'-nitro-Nitrosoguanidine (MNNG), nitrosoguanidine, 2-aminopurine, 7, 12 dimethyl-benz(a)anthracene (DMBA), ethylene oxide, hexamethylphosphoramide, bisulfan, diepoxyalkanes (diepoxyoctane (DEO), diepoxybutane (BEB), and the like), 2-methoxy-6-chloro-9[3-(ethyl-2-chloro-ethyl)aminopropylamino] acridine dihydrochloride (ICR-170), formaldehyde, and the like may be used to mutagenize the plant tissue in order to create the PG gene mutations of the present invention. Spontaneous mutations in the fruit PG gene that may not have been directly caused by the mutagen can also be identified using the present invention.

Any method of plant DNA preparation known to those of skill in the art may be used to prepare the tomato plant DNA for PG mutation screening. For example, See D.H. Chen and Ronald, P.C., *Plant Molecular Biology Reporter* 17: 53-57 (1999);

C.N. Stewart and Via, LE, *Bio Techniques*, 1993, Vol. 14(5): 748-749. Additionally, several commercial kits are available, including kits from Qiagen (Valencia, CA) and Qbiogene (Carlsbad, CA).

The prepared DNA from individual tomato plants are then pooled in order to expedite screening for mutations in the PG genes of the entire population of plants originating from the mutagenized plant tissue. The size of the pooled group is dependent upon the sensitivity of the screening method used. Preferably, groups of four or more individuals are pooled.

After the DNA samples are pooled, the pools are subjected to PG gene-specific amplification techniques, such as Polymerase Chain Reaction (PCR). For a general overview of PCR, see PCR Protocols: A Guide to Methods and Applications (Inns, M., Gelfand, D., Sninsky, J., and White, T., eds.), Academic Press, San Diego (1990). Any primer specific to the PG gene or the sequences immediately adjacent to the PG gene may be utilized to amplify the PG genes within the pooled DNA sample. Preferably, the primer is designed to amplify the regions of the PG gene where useful mutations are most likely to arise. For example, the primer should maximize the amount of exonic sequence of the PG gene and, likewise, avoid intronic sequences of the gene. Additionally, it is preferable for the primer to avoid known polymorphism sites in order to ease screening for point mutations. Furthermore, when specifically screening for mutations that will knock out the PG enzymatic activity, it is preferable to target the 5'-end of the PG gene or to target areas of the PG gene that are highly conserved. To facilitate detection of PCR products on a gel, the PCR primer may be labeled using any conventional labeling method. Exemplary primers (SEQ. ID.

Nos.3-46) that have proven useful in identifying useful mutations within the PG gene sequence are shown below in Table 1.

TABLE 1

NAME	SEQUENCE	SEQUENCE I.D. NO.
Lc_PG-L1	TTGAGACGGGAGAAGACAAGCCAGA	003
Lc_PG-L2	CCAACCATATGAACAACCTCACACATGC	004
Lc_PG-L3	TGTGGGGTAGATCGATCCAGAGGTTG	005
Lc_PG-L4	ACGCCTCGTACATTCGAGATCGTTG	006
Lc_PG-L5	TCACAAGAAAAGGGATAGTTCAAAGTG	007
Lc_PG-L6	TGAAGTCATTTCAAAACGAATCAAAT	008
LePG-L10-700	TTCTCCTTCTCATTATTATTTTTGCTTCATCA	009
LePG-L11-700	CTGGAATTGCAAAAATTTGAAAGTGAATAA	010
PG1Lnew-IRD	TTGAGACGGGAGAAGACAAGCCAGAC	011
PG3Lnew-IRD	AGTGGCTTTCGTACTACATAATCTTAG	012
PG-5Lnew	CATGCAATAATTATTGACGAAATGTGGT	013
PG-L1	TTGAGACGGGAGAAGACAAGCCAGA	014
PGL1 IRD700	TGAGACGGGAGAAGACAAGCCAGAC	015
PG-L10	TTCTCCTTCTCATTATTATTTTTGCTTCATCA	016
PG-L11	CTGGAATTGCAAAAATTTGAAAGTGAATAA	017
PGL12	TTGACGAAATGTGGTTTTGGTACCTATAATCTT	018
PGL14	CACAAACGAATACATGCAGATTCTCAAACA	019
PG-L2-700	CCAACCATATGAACAACCTCACACATGC	020
PG-L2B	ATCTTCAATCTACCATATTGAAATATTG	021
PG-L2C	TACATTTGGTAGTGTTTCTTATCGTG	022
PG-L3-new	AGTGGCTTTCGTACTACATAATCTTAG	023
PG-L7	CAAAAGACGAAATGATGAATAATTTTGCGAAT	024
PG-L8	CACAAACGAATACATGCAGATTCTCAAACA	025
PG-L8B	AGTAGAGTATATCCTTAAAAGAGAGC	026
PG-L9	ACGCCTCTGACATTCGAGATCGTTG	027
Lc_PG-R1	CCATGGAAAATAGCTTTTCCTCGCTTA	028
Lc_PG-R2	CATTTTGATAATTCCTCACTAATCCGCTAA	029
Lc_PG-R3	CAAGGGGTAATAGGTCCTGCCCAAA	030
Lc_PG-R4	CTGCTTTTATTCGCCCATCCAAACG	031
Lc_PG-R5	GAATCTCAAAGTTTTAATGATGTAAGGTGA	032
Lc_PG-R6	TTATACAAAAGAGCTTCATCCTCTGAAAT	033
PG-R10	CCTGTTGTATACATGGTTCAACTCGATCACA	034
PG-R11	CCTCTGAAATTTCTAGTGAAGTGCAGTGTGG	035
PG-R12	TCCATGGAAAATGACTTTCCTCGCTTAC	036
PG-R13	ATAGAAGATCTGCATGGACCTGAAAAGGTGA	037
PG-R14	AAGTAATATTTGTGGCCTGCACATTTGAG	038
PG-R15	CCTAATTATTGTGCTAAGTCATTAACCATAAAGAC	039
PGR16	GACCATAGTCCAAAAGATCCATAAATTAGAAGAAAA	040
PGR17	TGACATTATAGTTCAACAAGAAATACCAAAGGGATA	041
PG-R7	ACCATGGAAAATAGCTTTCCTCGCTTAA	042
PG-R8	CAAAGGGGTAATAGTCCTGCCCAAA	043
PG-R9	CTACTTTTATTACGCCCATCCAAACG	044
PGseqint7	AAGTGTAAATGTGTTGCTTTGTTTAGAAGTTTGG	045
Pgint8	TGAAAAGAATCTCAAAGTTTTAATGATGTAAGGTGA	046

The PCR amplification products may be screened for PG mutations using any method that identifies heteroduplexes between wild type and mutant genes. For example, but not limited to, denaturing high pressure liquid chromotography (dHPLC), constant denaturant capillary electrophoresis (CDCE), temperature gradient capillary electrophoresis (TGCE) (Q. Li, *et al.*, *Electrophoresis*, 23(10):1499-1511 (May 2002), or by fragmentation using chemical cleavage, such as used in the high throughput method described by Colbert *et al.*, *Plant Physiology*, 126:480-484 (June 2001). Preferably the PCR amplification products are incubated with an endonuclease that preferentially cleaves mismatches in heteroduplexes between wild type and mutant sequences. Cleavage products are electrophoresed using an automated sequencing gel apparatus, and gel images are analyzed with the aid of a standard commercial image-processing program.

Mutations that reduce PG enzyme activity in the plant are desirable. Preferred mutations include those that prematurely truncate the translation of the PG protein, such as those mutations that create a stop codon within the amino acid sequence of the PG protein. Additional preferred mutations include those that cause the mRNA to be alternatively spliced, such as mutations in and around the intron splice sites within the mRNA. Furthermore, any mutations that create an amino acid change within one of the fifteen highly conserved residues of the PG polypeptide are also preferred.

Once an M2 plant having a mutated PG gene is identified, then the mutations are analyzed to determine its potential affect on the expression, translation, and/or activity of the PG enzyme. First, the PCR fragment containing the mutation is sequenced, using standard sequencing techniques, in order to determine the exact location of the mutation in relation to the overall PG gene sequence. Second, in order

to determine the severity of the change, a LOGO analysis is performed on the amino acid sequence BLOCK in which a mutation is located. Protein BLOCKS are multiply-aligned, ungapped segments corresponding to the most highly conserved regions of the protein families. Henikoff *et al.*, *Gene* 163: GC17-GC26 (1995). LOGOs are a graphical representation of aligned sequences where the size of each amino acid residue is proportional to its frequency in that position. The LOGO for a BLOCK is calculated from the position-specific scoring matrix (PSSM). Tomato PG belongs to the glycoside hydrolase protein family 28 (BLOCK IPB000743). One hundred and forty-seven members of this family were used to identify the seven conserved blocks within the family that are included in the BLOCKS database.

If the initial assessment of the mutation in the M2 plant appears to be in a useful position within the PG gene, then further phenotypic analysis of the tomato plant containing that mutation is pursued. First, the M2 plant is backcrossed twice in order to eliminate background mutations. Then the M2 plant is self-pollinated in order to create a plant that is homozygous for the PG mutation.

Physical and biochemical characteristics of these homozygous PG mutant plants are then assessed. Mutant PG tomatoes are evaluated for delayed softening compared to the normal (wild type) parental tomato lines. Normal fruit ripens such that the color of the tomato changes from light green to red. As this change happens, the fruit tends to become softer such that compression under a specified weight becomes greater and/or the force required to depress the surface of the fruit a specified distance becomes greater. See Cantwell, M. Report to the California Tomato Commission: Tomato Variety Trials: Postharvest Evaluations for 2001; Edan, Y., H. Pasternak, I. Shmulevich, D. Rachmani, D. Guedalia, S. Grinberg and E.

Fallik. 1997. Color and firmness classification of fresh market tomatoes. J. Food Science 62(4): 793-796; Errington, N., J.R. Mitchell and G.A. Tucker. 1997. Changes in the force relaxation and compression responses of tomatoes during ripening: the effect of continual testing and polygalacturonase activity. Postharvest Biol. Tech. 11: 141-147; Lesage, P. and M-F. Destain. 1996. Measurement of tomato firmness by using a non-destructive mechanical sensor. Postharvest Biol. Tech. 8: 45-55.

The following mutations are exemplary of the tomato mutations created and identified according to the present invention. One exemplary mutation, correlates with a change of G to A at nucleotide 1969 of SEQ. ID. NO. 1, counting A in the ATG of the START CODON as nucleotide position 1. This mutation results in a change from glycine to arginine at amino acid 178 in the expressed protein. The change from glycine to arginine at 178 is a dramatic amino acid change both in terms of charge and size. The G178R mutation is within block B of this family. As shown in **FIG. 2**, G178 is one of the fifteen most conserved residues within the glycoside hydrolase protein family. *Lycopersicon esculentum* seeds of the cultivar Shady Lady containing this mutation were deposited with the American Type Culture Collection, 10801 University Blvd., Mannassas, VA 20110-2209, on September 9, 2002 and given Accession No. 13345 and Patent Deposit Designation PTA-4702.

Another exemplary mutation, created and identified according to the present invention, correlates with a T to A change at nucleotide position 2940 of SEQ. ID. NO. 1, counting A in the ATG of the START CODON as nucleotide position 1. This mutation results in a change from histidine to glutamine at amino acid 252. The H252Q mutation is within block D of the glycoside hydrolase protein family. As

shown in **FIG. 3**, H252Q is also a change in a very conserved region of this protein family. *Lycopersicon esculentum* seeds of the cultivar Shady Lady containing this mutation were deposited with the American Type Culture Collection, 10801 University Blvd., Mannassas, VA 20110-2209, on September 20, 2002 and given Accession No. 13342 and Patent Deposit Designation PTA-4702.

The following Examples are offered by way of illustration, not limitation.

EXAMPLE 1

Mutagenesis

In one embodiment of the present invention tomato seeds of cultivars Shady Lady (hybrid) and NC 84173 (inbred line provided by R. Gardner at the University of North Carolina) were vacuum infiltrated in H_2O (ca. 4 min. with ca. 1000 seeds/100 ml H_2O). The seeds were then placed on a shaker (45 rpm) in a fume hood at ambient temperature. The mutagen ethyl methanesulfonate (EMS) was added to the imbibing seeds for final concentrations ranging from about 0.1% to about 1.6% (v/v). EMS concentrations of about 0.4 to about 1.2% were determined to be optimal for these studies. Following a 24-hour incubation, the EMS solution was replaced with fresh H_2O (4x to an est. EMS dilution 1/2,000,000,000). The seeds were then rinsed under running water for ca. 1 hour. Finally, the mutagenized seeds were planted (96/tray) in potting soil and allowed to germinate in the greenhouse. Four to six week old surviving plants were transferred to the field to grow to fully mature M1 plants. The mature M1 plants were allowed to self-pollinate and then seeds from the M1 plant were collected and planted to produce M2 plants.

DNA Preparation

DNA from these M2 plants was extracted and prepared in order to identify which M2 plants carried a mutation in their PG gene. The M2 plant DNA was prepared using the methods and reagents contained in the Qiagen® (Valencia, CA) 96 Plant Kit. Approximately 0.1g of frozen plant sample was placed in a sample tube with a tungsten bead, frozen in liquid nitrogen and ground 2 times for 1 minute each at 20 Hz using the Qiagen® Mixer Mill MM 300. Next 400µl solution AP1 [buffer AP1, solution DX and RNAse (100µg/ml)] at 80°C was added to the sample. The tube was sealed and shaken for 15 seconds. Following the addition of 130 µl buffer AP2, the tube was shaken for 15 seconds. The samples were then frozen for at least 10 minutes at minus 20°C. The samples were then centrifuged for 20 minutes at 5600 X g. A 400µl aliquot of supernatant was transferred to another sample tube. Following the addition of 600µl of buffer AP3/E, this sample tube was capped and shaken for 15 seconds. A filter plate was placed on a square well block and 1ml of the sample solution was applied to each well and the plate was sealed. The plate and block were centrifuged for 4 minutes at 5600 X g. Next 800 µl of buffer AW was added to each well of the filter plate, sealed and spun for 15 minutes at 5600 X g in the square well block. The filter plate was then placed on a new set of sample tubes and 100 µl of buffer AE was applied to the filter. It was capped and incubated at room temperature for 1 minute and then spun for 2 minutes at 5600 X g. This step was repeated with an additional 100 µl buffer AE. The filter plate was removed and the filtrates were pooled and the tubes capped. Then the individual samples were normalized to a concentration of 25ng/µl.

TILLING

The M2 DNA was pooled into groups of four or more individual plants each. For pools containing four individuals, the DNA concentration for each individual within the pool was 0.25 ng/μl with a final concentration of 1ng/μl for the entire pool. The pooled DNA samples were arrayed on microtiter plates and subjected to gene-specific PCR.

PCR amplification was performed in 15μl volumes containing 5ng pooled or individual DNA, 0.75X ExTaq buffer (Panvera, Madison, WI), 2.6 mM MgCl2, 0.3mM dNTPs, 0.3μM primers, 0.05U Ex-Taq (Panvera, Madison, WI) DNA polymerase. PCR amplifications were performed using an MJ Research thermal cycler as follows: 95°C for 2 minutes; 8 cycles of "touchdown PCR" (94°C for 20 second, followed by annealing step starting at 70-68°C for 30 seconds decreasing 1°C per cycle, then a temperature ramp of 0.5°C per second to 72°C followed by 72°C for 1 minute); 25-45 cycles of 94°C for 20 seconds, 63-61°C for 30 seconds, ramp 0.5°C/sec to 72°C, 72°C for 1 minute; 72°C for 8 minutes; 98°C for 8 minutes; 80°C for 20 seconds; 60 cycles of 80°C for 7 seconds –0.3 degrees/cycle.

The PCR, primers (MWG Biotech, Inc., High Point, NC) were mixed as follows:

9μl 100μM IRD-700 labeled Left primer

1μl 100μM Left primer

10μl 100μM Right primer

The IRD-700 label can be attached to either the right or left primer. Preferably, the labeled to unlabeled primer ratio is 9:1. Alternatively, Cy5.5 modified primers or

IRD-800 modified primers could be used. The label was coupled to the oligonucleotide using conventional phosphoamidite chemistry.

For digestion of 15-μL PCR products in 96-well plates, 30 μL of a solution containing 10mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.5), 10 mM $MgSO_4$, 0.002% (w/v) Triton X-100, 20 ng mL^{-1} of bovine serum albumin, and 1/1000 dilution of CEL 1 (50 units μL^{-1}) was added with mixing on ice, and the plate was incubated at 45°C for 15 min. CEL 1 was purified from 30 kg of celery as described by Oleykowski *et al.*, *Nucleic Acids Res* 26: 4597-4602 (1998), except that Poros HQ rather than Mono Q was used, and the PhenylSepharose and Superdex 75 columns were omitted. The specific activity was 1 x 10^6 units mL^{-1}, where a unit is defined as the amount of CEL 1 required to digest 50% of 200 ng of a 500-bp DNA fragment that has a single mismatch in 50% of the duplexes. Reactions were stopped by addition of 5 μL 0.15 M EDTA (pH 8) and the mixture pipetted into wells of a spin plate (G50, Sephadex) prepared and spun according to the manufacturer's recommendations into a plate containing 1 to 1.5 μL of formamide load solution [1mM EDTA (pH 8) and 200 μg mL^{-1} bromophenol blue in deionized formamide]. The volume was reduced to a minimum by incubation at 80°C uncovered (30-40 min) and stored on ice, then transferred to a membrane comb using a comb-loading robot (MWG Biotech). Alternatively, the DNA samples could have been concentrated using isopropanol precipitation. The comb was inserted into a slab acrylamide gel, electrophoresed for 10 min, and removed. Electrophoresis was continued for 4h at 1,500-V, 40-W, and 40-mA limits at 50°C.

After electrophoresis, the gel was imaged using a LI-COR (Lincoln, NE) scanner which was set at a channel capable of detecting the IR Dye 700 label. The gel

image showed sequence-specific pattern of background bands common to all 96 lanes. Rare events, such as mutations, created a new band that stood out above the background pattern. Plants with bands indicative of mutations of interest were evaluated by sequencing individual PCR products. Plants carrying mutations confirmed by sequencing were grown up as described above (e.g., the M2 plant was backcrossed twice in order to eliminate background mutations and self-pollinated in order to create a plant that was homozygous for the mutation).

Physical and Biochemical Measurements

Tomatoes Selected for Study:

Individual tomatoes selected for study were picked from plants derived from siblings of the same cross to preserve background phenotypes as much as possible. The plants and fruit were genotyped as homozygous for the mutation, heterozygous for the mutation, or wild type. Genotyping was performed using a genetic method for determining single base pair mismatches referred to in the scientific literature as "dCAPing", see M.M Neff et al., *The Plant Journal* 14:387-392 (1998). Briefly, a degenerate PCR oligonucleotide is designed to create a restriction endonuclease recognition site when the mutant base pair is present. Plants are then simply genotyped using a PCR reaction followed by a restriction enzyme digestion and then analysis on an agarose gel.

Squeeze Test:

A test was devised to simulate consumer perception of tomato fruit firmness in the three genotypes of the 13345 mutant. Fruits were evaluated at the red ripe stage. Four fruits of each genotype were blindly labeled, and 15 people were asked to rank each set as most firm, least firm, or in between (mid firm). Of the people surveyed,

80% ranked the homozygous PG mutant tomatoes as the most firm; 20% ranked the heterozygous mutant tomatoes as the most firm; and no one ranked the wild type as the most firm. Results are shown in **FIG. 4**.

Color Determination:

Objective color values were determined for table-ripe wild type and mutant 13345 and 13342 tomatoes using a Minolta Color meter. Data were reported as "hue" and from 20-30 hue values were measured. Hue is the single most useful color value and the lower the hue value, the redder the tomato. In support of the idea that some characteristics associated with ripening do not differ, the results showed that PG mutants (hue values of 35.8 and 34.5) were similar in color to wild type tomatoes (hue value of 36.6).

Assays for PG Activity:

Polygalacturonase enzymatic activity was measured spectrophotometrically using two different in vitro color assays that quantify the formation of reduced sugars from a polygalacturonic substrate. One assay utilized 3,5-dintrosalicylate (DNS) for color detection, and was performed as in Redenbaugh K, Hiatt W, Martineau B, Kramer M, Sheehy R, Sanders R, Houck C, and Emlay D. Safety Assessment of Genetically Engineered Fruits and Vegetables: A Case Study of the Flavr Savr Tomato. CRC Press (1992); R. Sheehy, et al., *PNAS* 85:8805-8809 (1988); Z. M. Ali and C.J. Brady, *Aust. J. Plant Physiol.* 9:155-169 (1982). The other assay utilized bicinchoninic acid (BCA) as the color substrate and was performed as in G.E. Anthon et al., *Journal of Agricultural and Food Chemistry* 50:6153-6159 (2002); and D. Fachin, et al., *Journal of Food Science* 67:1610-1615 (2002).

DNS Based Assay for PG Activity: Briefly, cell wall extracts from individual tomatoes were prepared as follows: tomatoes were sliced, locular tissue and seeds were removed, and 100 grams of the remaining tomato tissue were homogenized in 300 milliliters (ml) cold H_2O and centrifuged at 4000 rpm in a tabletop centrifuge. The pellet was resuspended in 300 ml extraction buffer (1.7 M NaCl, 40mM ß-mercaptoethanol, 50mM sodium phosphate, pH 4.6) and stirred for 4 hours at 4° C. The suspension was then centrifuged as before and the supernatant was reserved for use in the DNS color assay. Because PG enzyme is the predominant protein in the cell wall extracts, any variation in PG protein amount due to genotype would interfere with using protein concentration in the normalization process, thus in the 13345 mutant where PG protein is absent the lysates were instead normalized to wet weight of starting material.

For the color assay, 0.1 ml 2M ammonium chloride, 1 ml 1% polygalacturonic acid, and 0.1 ml cell wall extract were mixed together in tubes on ice. Samples were vortexed and a small amount was reserved as a control for the amount of reduced sugars present prior to incubation with PG enzyme. The remainder of each sample was incubated at 37° C for 2 hours. After incubation, samples were place on ice and 0.1 ml of each was transferred to a new tube at room temperature with 0.2 ml DNS color reagent (1g DNS/20 mls 2M NaOH, 30 g sodium potassium tartarate/50mls warm water; the two reagents are then combined and diluted to 100 mls with warm water). Tubes were boiled in a water bath for 5 minutes and then 2 mls H_2O added to each tube. Tubes were spun to clarify and then read at an absorbance of A540 on a spectrophotometer.

Results of the DNS based PG activity assay, shown in **FIG. 5**, demonstrate that homozygous 13345 tomato fruits have less than 40% the activity of the wild type control. Tomatoes used in this assay were vine ripened and picked at equivalent stages in development.

BCA Based Assay for PG Activity: Briefly, cell wall extracts from individual tomatoes were prepared as follows: tomatoes were sliced, locular tissue and seeds were removed, and 15 g of the remaining tissue was homogenized in 30ml cold H_2O. ~7.5 mls 1N HCl was added (to a final pH of 3.0), and the homogenate was spun at 4000 rpm in a tabletop centrifuge. The pellet was washed in 30 mls cold H_2O, and spun as before, The washed pellet was then resuspended in 7.5 mls extraction buffer (0.1M sodium phosphate pH 6.5, 1.2M NaCl) and incubated on ice for 30 minutes. The suspension was spun as before, and the supernatant was reserved for use in the BCA color assay. Again, the lysates were normalized to wet weight starting material in the 13345 mutant, and protein concentration in the 13342 mutant.

For the BCA color assay, 0.5 ml 1% polygalacturonic acid, 0.2 ml 1M NaCl, 1.3 ml H_2O, and 10μL extract were mixed together and incubated at 37° C for 30 minutes. After incubation, 1 ml carbonate buffer (54.3g/L disodium carbonate/24.2 g/L sodium monocarbonate) was added to terminate each reaction. 0.6 mls each terminated reaction was then added to 1.9 ml H_2O and 1.6 mls color reagent (equal volumes reagents A and B where reagent A is 1.96 g bicinchoninic acid/L H_2O and reagent B is 1.24 g/L $CuSO_4$-H_2O, 1.26 g/L L-serine), and incubated at 80° C for 30 minutes. Color development was then measured at A560 using a spectrophotometer.

Results of the BCA based PG activity assay, shown in **FIG. 6**, demonstrate that both mutants exhibit decreased PG activity as compared to wild type (controls).

For the 13342 mutant, tomatoes from both M3 and F2 generations of tomatoes were assayed. For the 13345 mutant, tomatoes from M3, F2 and F3 generations were assayed. These assays not only demonstrate efficacy of the mutations in decreasing PG enzymatic activity, they also demonstrate the stability of the mutations in a breeding program.

Western Blot:

To ascertain the amounts of PG enzyme in the mutant tomatoes relative to wild type tomatoes, cell wall extraction lysates from the activity assays were run on SDS-PAGE gels and visualized both by Coomassie stain and by Western blot using a PG-specific polyclonal antibody as in D. DellaPenna et al., *PNAS*, 83:6420-6424 (1986). (PG antibody was a generous gift of Dr Alan Bennett, University of California, Davis).

Results of the Western blot, shown in **FIG. 7,** demonstrate that significantly less PG protein is detected in cell wall extraction lysates from mutant 13345 tomatoes than from wild type controls. The level of PG protein detected in red ripe mutant 13345 tomatoes is approximately that found in the early developmental stages of wild type tomatoes (**FIG. 8**). As shown in **FIG. 9**, the level of PG protein detected in red ripe mutant 13342 tomatoes is approximately the same as that found in red ripe wild type tomatoes. The Western blot results combined with the PG enzyme activity data for mutant 13342 tomatoes indicate that a non-functional form of PG protein is present in mutant 13342 tomatoes. Coomassie staining shows that PG is the predominant protein found in cell wall extraction lysates.

Identification and Evaluation of Mutation 13345

DNA from tomato plant 13345, originating from seeds of cultivar Shady Lady that were incubated in 1.2% EMS, was amplified using primer pair PGL3 (SEQ. ID. NOs. 023 and 043). The PCR amplification products were then incubated with CEL 1 and electrophoresed. The electrophoresis gel image showed a fragment at the approximate position of 204bp, above the background pattern for the PCR amplification products. Therefore, it was likely that this fragment contained a heteroduplex created by a mutation in the PG gene. Sequence analysis of this fragment showed that the mutation was associated with a G to A change at nucleotide 1969 of SEQ. I.D. No. 1, counting A in the ATG of the START CODON as nucleotide position 1. This mutation correlates with a change from glycine to arginine at amino acid 178 of the PG polypeptide.

This mutation is within block B of the glycoside hydrolase protein family. LOGO analysis of the G178R mutation within this block revealed that the mutation lies at one of the fifteen most conserved amino acids within the family.

Tomato fruits containing Mutation 13345 exhibited lower PG enzyme activity compared to their wild type sibling, and were considered firmer than the wild type sibling.

Identification and Evaluation of Mutation 13342

Tomato plant 13342, originating from seeds of cultivar Shady Lady that were incubated in 0.6% EMS, was screened with primer pair PGL9 (SEQ. ID. NOs. 027 and 039). The PCR amplification products were then incubated with CEL 1 and

electrophoresed. The electrophoresis gel image showed a fragment at the approximate position of 385bp, above the background pattern for the PCR amplification products. Therefore, it was likely that this fragment contained a heteroduplex created by a mutation in the PG gene. Sequence analysis of this fragment showed the mutation was associated with a T to A change at nucleotide 2940 of SEQ. I.D. No. 1, counting A in the ATG of the START CODON as nucleotide position 1. This mutation correlates with a change from histidine to glutamine at amino acid 252 of the PG polypeptide.

Tomato fruits containing Mutation 13345 exhibited lower PG enzyme activity compared to their wild type sibling, and were consider former than their wild type sibling.

The above examples are provided to illustrate the invention but not limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims and all their equivalents. All publications, patents, and patent applications cited herein are hereby incorporated by reference.